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We found that Ras was highly activated in 11 of 20 breast cancers compared to normal tissue; 7 of these 11 cancers expressed both the epidermal growth factor (EGF) and ErbB-2/*neu*/HER-2 receptors with the remaining four cancers with high Ras activation expressing one of these two receptors. In the other 9 cancers, Ras activation was similar to that observed in normal breast tissue with none of these cancers expressing the EGF receptor while one expressed the ErbB-2 receptor. None of the cancers tested had an activating K-*ras* mutation nor did any of the cancers express a truncated EGF receptor or the c-FMS receptor. The activity of mitogen-activated protein (MAP) kinase was high in the cancers and reflected the degree of Ras activation. In cultured mammary tumor cell lines, we showed that Ras activation was ligand dependent in cells overexpressing the ErbB-2 receptor. Thus, Ras was abnormally activated in breast cancers overexpressing the EGF and/or ErbB-2 receptors indicating there are sufficient ligands *in vivo* to activate these receptors and this work provides a basis for new target-based treatments of this disease. Using a newly-developed assay, we are now in the process of measuring the activation state of Rho, a Ras-related protein that has been shown to play a role in carcinogenesis.

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INTRODUCTION

Ras, the product of the *ras* proto-oncogene, alternates between an active GTP-bound state and an inactive GDP-bound state and transmits growth-promoting signals from the plasma membrane to the nucleus (1). Activating mutations of codons 12, 59 or 61 of Ras can lead to malignant transformation and are found in a variety of human cancers, including about 90% of pancreatic cancers, 50% of colon cancers, 50% of thyroid cancers and 5% of breast cancers (1; 2). The low percentage of breast cancers with activating *ras* mutations has led to the notion that Ras does not play an important pathogenetic role in this cancer (3). However, several growth factor receptors which signal through Ras are overexpressed in breast cancer suggesting that Ras may be activated in some breast cancers through upstream mechanisms (4-10). Two of these growth factor receptors are the epidermal growth factor (EGF) receptor and the ErbB-2/*neu*/HER-2 receptor; they are overexpressed in 20-50% of human breast cancers and their increased expression predicts shortened disease-free survival and overall survival (4; 5; 11; 12). In addition, a truncated constitutively-active EGF receptor has been reported in breast cancer and the c-FMS/colony stimulating factor-1 (CSF-1) receptor, which also signals through Ras, is expressed in ~15% of breast cancers but not in normal breast tissue (13-15). Although high expression of the EGF and ErbB-2 receptors correlates with a poor prognosis in breast cancer, it is not known whether sufficient ligands are present in breast tissue to activate the receptors and which of the signal transduction pathways activated by the receptors in cultured cells is/are activated in primary cancers (4; 5; 11; 12).

BODY

We have submitted a manuscript describing the first 20 breast cancer samples analyzed for Ras activation and EGF and ErbB-2 receptor expression; the breast cancers were compared to seven normal breast samples and two fibroadenomas (manuscript is included in the Appendix). We defined increased Ras activation as activation levels of greater than two standard deviations above the mean of the seven normal samples. In the breast cancers there was a remarkable correlation between increased Ras activation and overexpression of the EGF and/or ErbB-2 receptors: 11 of the breast cancers showed increased Ras activation and seven of these overexpressed both the EGF and ErbB-2 receptors while the other four with increased Ras activation overexpressed one of these two receptors. This is in stark contrast to the nine cancers with normal Ras activation levels in which none overexpressed the EGF receptor and only one overexpressed the ErbB-2 receptor. Thus, there was a high correlation between increased expression of the EGF and/or ErbB-2 receptors and increased Ras activation. None of the breast cancers had a K-*ras* activating mutation nor did any of the cancers express the truncated constitutively active EGF receptor or the c-FMS/CSF-1 receptor. In the cancers with increased Ras activation, there was increased activation of mitogen-activated protein (MAP) kinase, thus indicating that Ras signals thru the Raf kinase, MEK, MAP kinase pathway in breast cancer. The data from the primary cancers correlated well with data from three different breast cancer cell lines, one without any known genetic mutations, one with an activating K-*ras* mutation and one which overexpressed the ErbB-2 receptor. Specifically, the degree of Ras activation was similar in the cancers with high Ras activation as in the cell line with the K-*ras* mutation and the cell line overexpressing the ErbB-2 receptor. Ras activation levels in the cancers with low Ras activation were similar to that found in the cell line without any known genetic

mutations. In the cell line overexpressing the ErbB-2 receptor, growth factors were required to activate Ras; since we found Ras to be highly activated in cancers overexpressing the EGF and/or ErbB-2 receptors, this indicates there are sufficient ligands in breast tissue *in vivo* to activate Ras. Thus, this work provides a strong basis for treating selected breast cancers with inhibitors of the Ras/MAP kinase pathway. In addition to pharmacologic agents, there are antibodies and oncolytic viruses which target the Ras/MAP kinase pathway suggesting that these various approaches could be of value in the treatment of breast cancer (16; 17). As part of another research project, we are developing an instrument to automate our method for measuring Ras activation in primary cancers and the prototype instrument should be completed within the next several months. Ultimately, this will allow measuring Ras activation in clinical laboratories and the work funded by the present grant proposal will, therefore, represent the initial studies of Ras activation in breast cancer.

We are continuing to measure Ras activation in breast cancers and would anticipate that by the completion of the grant period in one year hence we will have performed this measurement on about 40 cancers. In addition to assessing Ras activation in the recent cancers, we have begun to assess the activation state of Rho, a Ras-related protein. Rho proteins are necessary for *ras*-transformation and regulate cell morphology, adhesion and motility through cytoskeletal dynamics and play an important role in carcinogenesis (18). Rho proteins are activated by serum stimulation and adhesion to fibronectin; they are involved in the changes in cell attachment and migration that are required for tumor cell invasion and metastasis (19; 20). Thus, we decided to assess Rho activation in primary and metastatic breast cancers. In order to perform these measurements, we first had to develop an assay for measuring Rho activation. Unlike for Ras, a good immunoprecipitating antibody against Rho is not available. Thus, we have used the Rho binding domain of the Rho effector Rhotekin (21; 22). This protein binds with a very high affinity to activated Rho, i.e., Rho in the GTP-bound state but not to Rho in the unactivated GDP-bound state. In primary breast samples, measuring active Rho would not be sufficient since tissue heterogeneity could lead to markedly different amounts of Rho in different samples. We, therefore, devised a method to measure GDP-bound Rho which takes advantage of the fact that the exchange of GTP for GDP on Rho takes place very quickly in the absence of magnesium. Thus, we split the samples in half and incubate one half with exogenous GTP; since we extract the cells in Triton X-114, excess GTP can be removed by three rapid and successive phase extractions. We have found this method to be highly accurate in pilot studies performed on the breast cancer cell lines described above. To date, we have assessed Rho activation in benign breast tissue from two subjects and in two breast cancers. Interestingly, Rho was much more activated in the two cancers than in the two normal breast tissues. Clearly, much more work needs to be done using this newly-developed technology. Recent work has shown that farnesyl transferase inhibitors, which were originally developed as Ras inhibitors, may actually function by inhibiting Rho (23; 24). Thus, assessing a tumor for its degree of Rho activation may provide data concerning whether a farnesyl transferase inhibitor would be of use in treating the cancer. Determining which breast cancers might be treated with a farnesyl transferase inhibitor was one of the objectives of the original grant application.

KEY RESEARCH ACCOMPLISHMENTS

- Ras is activated in primary human breast cancers in the absence of a mutation in the *ras* gene
- Ras activation occurs in tumors with overexpression of the EGF and/or ErbB-2 receptors
- Ras activation in breast cancer correlates with MAP kinase activity
- In a human breast cancer cell line overexpressing the ErbB-2 receptor, Ras activation occurred only when the cells were exposed to sufficient ligand
- In breast cancers overexpressing the EGF or ErbB-2 receptors, there are sufficient ligand *in vivo* to activate the receptors and, thereby, activate Ras.

REPORTABLE OUTCOMES

A manuscript has been submitted describing the results to date; it is provided in the Appendix.

CONCLUSIONS

This work shows definitively that Ras is activated in approximately half of all human breast cancers. This is an important finding because it indicates that patients who have tumors with high Ras activation are candidates for therapies aimed at Ras or other protein targets in the Ras/MAP kinase pathway. Once the method for measuring Ras activation becomes automated, assessing Ras activation in tumors may become important clinical information.

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Ras Activation In Human Breast Cancer[1]

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ABSTRACT

Although genetic *ras* mutations are infrequent in breast cancer, Ras may be pathologically activated in breast cancer by overexpression of growth factor receptors which signal through Ras. We measured Ras activation in 20 breast cancers and found that in 11 of the cancers, Ras was highly activated compared to normal tissue; 7 of these 11 cancers expressed both the epidermal growth factor (EGF) and ErbB-2/*neu*/HER-2 receptors with the remaining four cancers with high Ras activation expressing one of these two receptors. In the other 9 cancers, Ras activation was similar to that observed in normal breast tissue with none of these cancers expressing the EGF receptor while one expressed the ErbB-2 receptor. None of the cancers tested had an activating K-*ras* mutation nor did any of the cancers express a truncated EGF receptor or the c-FMS receptor. The activity of mitogen-activated protein (MAP) kinase was high in the cancers and reflected the degree of Ras activation. In cultured mammary tumor cell lines, we showed that Ras activation was ligand dependent in cells overexpressing the ErbB-2 receptor. Thus, Ras was abnormally activated in breast cancers overexpressing the EGF and/or ErbB-2 receptors indicating there are sufficient ligands *in vivo* to activate these receptors and this work provides a basis for new target-based treatments of this disease.

INTRODUCTION

Ras, the product of the *ras* proto-oncogene, alternates between an active GTP-bound state and an inactive GDP-bound state and transmits growth-promoting signals from the plasma membrane to the nucleus (1). Activating mutations of codons 12, 59 or 61 of Ras can lead to malignant transformation and are found in a variety of human cancers, including about 90% of pancreatic cancers, 50% of colon cancers, 50% of thyroid cancers and 5% of breast cancers (1; 2).

The low percentage of breast cancers with activating *ras* mutations has led to the notion that Ras does not play an important pathogenetic role in this cancer (3). However, several growth factor receptors which signal through Ras are overexpressed in breast cancer suggesting that Ras may be activated in some breast cancers through upstream mechanisms (4-10). Two of these growth factor receptors are the EGF[1] and the ErbB-2/*neu*/HER-2 receptor; they are overexpressed in 20-50% of human breast cancers and their increased expression is associated with shortened disease-free survival and overall survival (4; 5; 11; 12). In addition, a truncated constitutively-active EGF receptor, ΔEGFR which lacks 267 amino acids in the receptor's extracellular domain, has been reported in breast cancer and the c-FMS/colony stimulating factor-1 receptor, which also signals through Ras, is expressed in ~15% of breast cancers but not in normal breast tissue (13-15). While high expression of the EGF and ErbB-2 receptors are poor prognostic factors in breast cancer, it is not known whether sufficient ligands are present in breast tissue to activate the receptors and which of the signal transduction pathways activated by the receptors in cultured cells is/are activated in primary cancers (4; 5; 11; 12). Increased activity of MAP kinase has been reported in breast cancer (16), but this enzyme can be activated by more than one mechanism and Ras activates more than MAP kinase in transmitting pro-proliferative signals (17; 18).

We investigated the activation state of Ras in 20 human breast cancers and compared the data to those obtained in seven normal breast samples, two fibroadenomas and three cultured human breast cancer cell lines, one without any known genetic mutations, one which overexpresses the ErbB-2 receptor and one which has an activating K-*ras* mutation. When compared to normal breast tissue, 11 of the 20 breast cancers demonstrated a two to six-fold increase in Ras activation and greater than a five-fold increase in MAP kinase activity; all 11 of these cancers showed increased expression of the EGF and/or ErbB-2 receptors. The degree of Ras activation in the 11 cancers was similar to that observed in the ErbB-2-overexpressing cell line treated with growth factors and in the cell line with a K-*ras* mutation under all culture conditions. Because Ras is the target of new pharmacologic and viral oncotherapeutic agents, this work provides a rationale for novel treatment strategies of breast cancer.

METHODS

Harvesting of Human Breast Tissue For Measurement of Ras Activation

Human breast tissue was obtained according to a procedure approved by the UCSD IRB. The pathologist sectioned routine surgical specimens and applied tissue scrapings to glass slides on dry ice; approximately 1 X 10^6 cells were obtained per slide and cells were frozen between 5 and 15 min of surgical resection. Only samples which yielded >80% epithelial cells were analyzed further; Fig. 1 shows representative scrapings from normal and malignant tissue stained histochemically and with an anti-keratin antibody. By taking serial sections every 5 min from a large tumor, we showed that Ras activation is stable if cells are frozen within 20 min of surgical resection; in addition, by comparing freshly harvested samples to frozen samples, we showed that slides can be stored at -80°C for several months without any change in Ras activation. There was no preselection of patient samples; however, there was an inherent bias toward tumors of ≥ 1 cm in size because they were more likely to be found quickly and provide sufficient material.

Measurement of Ras Activation

Ras activation is defined as the percent of Ras molecules in the active GTP-bound state, i.e., (Ras-bound GTP/Ras-bound GDP + Ras-bound GTP) X 100, and was measured as previously described using enzyme-based methods (19-22). Briefly, frozen cells from two to three glass slides were lysed and either the rat monoclonal antibody Y13-259 (experimental sample) or nonspecific rat IgG (control sample) was added to the lysate; antibody Y13-259 is a pan-Ras antibody which recognizes all three forms of Ras, i.e., H-, K- and N-Ras. GTP and GDP were eluted from the immunoprecipitated Ras by heating to 100° C for 3 min. We have found that a 1 h incubation of the primary antibody (Y13-259) with cell extracts is sufficient to quantitatively immunoprecipitate Ras

(19); magnesium ion and high salt present in the buffer inhibit GTP/GDP dissociation from Ras and RasGAP activity, respectively, and antibody Y13-259 is a Ras neutralizing antibody which inhibits RasGEF and RasGAP from interacting with Ras (23). We have shown that antibody Y13-259 is superior to antibody Y13-238 for immunoprecipitating Ras, even though the latter antibody could, theoretically, precipitate Ras bound to its downstream effectors (24). We have shown in experiments where Ras was immunoprecipitated from cells previously incubated with $^{32}PO_4$ that the heating step quantitatively elutes GDP and GTP from Ras; in addition, we have shown by high performance liquid chromatography that at neutral pH less then 5% of GTP is destroyed when heated to 100°C for up to 10 min (19).

GTP was measured by converting it to ATP using the enzyme nucleoside diphosphate kinase (Sigma, St. Louis, MO) and ATP was measured by the luciferase/luciferin system according to the following reactions:

$$\text{GTP} + \text{ADP} \xrightarrow{\text{nucleoside diphosphate kinase}} \text{GDP} + \text{ATP}$$

$$\text{ATP} + \text{luciferin} \xrightarrow{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{pyrophosphate} + \text{light}$$

GDP was measured by converting it to GTP using pyruvate kinase and phosphoenolpyruvate with the GTP measured as described above:

$$\text{GDP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{GTP} + \text{pyruvate}$$

Both assays are sensitive to 1 fmol of nucleotide. When GTP is measured in the second step of the GDP assay, the sum of GTP + GDP is determined; thus, the amount of GTP in the sample must be subtracted from the amount of GTP + GDP to yield the amount of GDP.

The amounts of GDP and GTP in the samples were determined by subtracting the control sample from the experimental sample and then comparing the results with standard curves prepared with each sample set; the data are expressed as fmol of GTP or GDP per microgram of DNA.

Assessment for Activating Mutations in K-*ras*, Codon 12

DNA was isolated from frozen tissue or from paraffin blocks using a commercial kit (Puregene, Minneapolis, MN) and activating mutations in K-*ras*, codon 12 were detected by polymerase chain reaction (PCR)/restriction fragment length polymorphism using ~400 ng DNA template (25). PCR amplification of K-*ras* exon I sequences generates a DNA fragment of 157 bp; digestion of wild type sequences with the restriction enzyme BstNI yields fragments of 114, 29 and 14 bp whereas sequences containing a codon 12 mutation, first or second position, are cleaved only once resulting in fragments of 143 and 14 bp (25). A human pancreatic cancer with a K-*ras*, codon 12 mutation and the human breast cancer cell line MCF-7 containing wild type K-*ras* served as controls.

Immunohistochemistry

Paraffin-embedded tissue was incubated with a rabbit anti-EGF receptor polyclonal antibody (Santa Cruz Biotechnology), a rabbit anti-ErbB-2 receptor polyclonal antibody (Dako), a mouse anti-ΔEGFR monoclonal antibody (provided by A. Jungbluth of the Ludwig Institute, New York, NY), a rabbit anti-cFMS receptor polyclonal antibody (Santa Cruz Biotechnology), or a rabbit anti-keratin polyclonal antibody (Dako); the anti-ΔEGFR antibody does not cross-react with the full-length wild type EGF receptor (22). Antibodies were detected using an alkaline phosphatase kit (Dako) and slides were counterstained with nuclear fast red. Staining of >20% of the cells on the plasma membrane was considered positive. Immunohistochemical analysis of EGF and ErbB-2 receptor expression correlates well with results from Northern, Southern and Western blotting and ligand-binding assays (9; 11).

Measurement of MAP Kinase Activity

MAP kinase activity was measured by following phosphorylation of myelin basic protein in MAP kinase immunoprecipitates as previously described (24). Data are expressed as pmol/min/mg protein and the assay was linear with time and protein concentration.

Breast Cancer Cell Lines

The human breast cancer cell lines MCF-7, MDA-MB-453 and MDA-MB-231 were obtained from the American Type Culture Collection. Cells were grown in D-MEM/F-12 medium containing 8% transferrin-enriched calf serum and 2% fetal bovine serum (FBS). For measurement of Ras and MAP kinase activation under logarithmic growth, cells were harvested in mid-logarithmic phase and for growth factor stimulation experiments, cells were starved for 72 h in D-MEM/F-12 without serum and then incubated for 5 min with D-MEM/F-12 containing either 20% FBS or 100 ng/ml EGF. In some experiments, the anti-ErbB-2 monoclonal antibody 9G6 (Santa Cruz Biotechnology) was added at the time of transfer to serum-free medium. At the time of harvest, cells were washed once with ice-cold phosphate-buffered saline, scraped from the plates and collected by centrifugation. Cell pellets were frozen immediately on dry ice and kept at -80°C until extracted for Ras activation or MAP kinase activity.

Measurement of DNA and Protein

DNA was measured by fluorescence using bisbenzimidazole (26) and protein was measured by the Bradford method (27).

Statistical Evaluation of Data

Comparison of data groups was done by the two-tailed Student test, with a p value of <0.05 considered significant.

RESULTS

Clinical and Pathological Data

The clinical and pathological data of the patient cohort are shown in Tables I-III; Ras activation is included to allow direct comparison between the tables and Fig. 2.

We obtained normal breast epithelial cells from seven subjects: in four cases from a reduction mammoplasty with three of the patients previously having had a cancer in the contralateral breast and in three cases at the time of mastectomy for a cancer (Table I); in patient 3, the cancer was also analyzed (Table III). In addition, two fibroadenomas were analyzed (Table I).

We studied breast cancers from 20 patients and divided these patients into two groups based on Ras activation (described below). The age range and clinical stage and tumor size, grade and histology were similar in the two groups as were the percent cells in S phase, chromosomal ploidy and estrogen and progesterone receptor status (Tables II and III).

Ras Activation in Human Breast Tissue

In the seven normal breast samples, Ras activation varied between 1.0 and 6.8% with a mean value of 4.5% (Table I and Fig. 2). This degree of Ras activation is similar to what we have found in other normal human tissues including brain (21), peripheral nerve (20), and ovarian epithelium (unpublished data). Ras activation in the two fibroadenomas was similar to the normal breast tissue (Table I and Fig. 2).

Ras activation in the 20 breast cancers varied between 1.5 and 29% (Tables II and III and Fig. 2). In nine of the cancers, Ras activation was within two standard deviations of the mean of the normal breast samples (0.3-8.7%) and these nine breast cancers were combined as Group A in Table II and Fig. 2; two of these cancers were from a lymph node metastasis with the primary lesion

unavailable. In the remaining 11 cancers, Ras activation exceeded two standard deviations above the mean of the normal samples and these cancers were combined as Group B in Table III and Fig. 2. The mean Ras activation for the 11 Group B cancers was 17%, or approximately four times the mean Ras activation of the normal breast samples. The increased Ras activation in the Group B cancers was unlikely due to a generalized increase in Ras activation in the breast tissue of these patients because normal breast tissue from patient 3 exhibited similar Ras activation as in the other normal samples (Table I).

Assessment for Activating Mutations in K-*ras*, Codon 12

Increased Ras activation in the 11 Group B cancers could be secondary to an activating mutation in one of the three *ras* genes. Activating *ras* mutations are infrequent in breast cancer (<5%) and occur almost exclusively in K-*ras*, codon 12 (2; 28; 29). We found no K-*ras*, codon 12 mutations in seven Group A cancers (patients 10, 11, 12, 14, 15, 16 and 17) or eight Group B cancers (patients 3,19, 20, 21, 22, 23, 26 and 28), including cancers with Ras activation >20% (Fig. 3 shows five representative samples). Results are not available for two Group A and three Group B cancers because of insufficient tissue. As expected, normal breast tissue and the fibroadenomas expressed wild type *ras*.

Expression of the EGF, ErbB-2 and c-FMS Receptors

Since none of the breast cancers examined showed a K-*ras* mutation, increased Ras activation in these cancers could be secondary to overexpression of the EGF, ErbB-2 or c-FMS receptor. Receptor expression was assessed independently by two immunopathologists without knowledge of the samples' Ras activation. Fig. 4 shows representative examples of ErbB-2 and EGF receptor staining and cumulative results for EGF and ErbB-2 receptor expression are shown in Fig. 2. All

normal breast tissues were negative for expression of the EGF receptor while two normal breast samples expressed the ErbB-2 receptor (patients 2 and 4, Table I); in a recent study, 40 of 291 benign breast samples expressed the ErbB-2 receptor (30). Neither of the two fibroadenomas expressed the EGF or ErbB-2 receptor. None of the Group A cancers expressed the EGF receptor while one expressed the ErbB-2 receptor. Nine of the 11 Group B cancers expressed the EGF receptor and nine expressed the ErbB-2 receptor. None of the samples expressed the c-FMS receptor nor the constitutively-active ΔEGFR although we found positive membrane staining for these two receptors in human lymphoid tissue and in the U87MG glioblastoma cell line, respectively (22).

Thus, of the 11 Group B breast cancers with high Ras activation, seven co-expressed both the EGF and ErbB-2 receptor and the remaining four expressed either the EGF or ErbB-2 receptor. This is in contrast to the Group A cancers with low Ras activation where one of nine expressed the ErbB-2 receptor.

MAP Kinase Activity in Human Breast Tissue

To determine if high Ras activation led to high MAP kinase activity, we measured MAP kinase activity in samples with sufficient tissue (Fig. 5). MAP kinase activity in the seven normal breast samples, one fibroadenoma, and three Group A breast cancers was 0.09 ± 0.05, 0.18, and 0.20 ± 0.13 pmol/min/mg protein, respectively. Although the mean MAP kinase activity was ~2-fold higher in the breast cancers than in the normal samples, the difference between the two groups was not statistically significant.

In six Group B breast cancers, mean MAP kinase activity was 0.52 ± 0.22 pmol/min/mg protein, which was significantly higher ($p<0.01$) than in normal tissue. Therefore, samples with high Ras activation had high MAP kinase activity and the ~4-fold higher Ras activation in the Group B cancers

compared to normal breast tissue was reflected in a >5-fold increase in MAP kinase activity.

Ras Activation and MAP Kinase Activity in Human Breast Cancer Cell Lines

We examined the effect of growth factors on Ras activation and MAP kinase activity in three well-characterized breast cancer cell lines: MCF-7 cells lacking known genetic mutations in Ras or growth factor receptors; MDA-MB-453 cells which overexpress the ErbB-2 receptor; and MDA-MB-231 cells which contain an activating K-*ras*, codon 12 mutation.

In serum-containing medium, Ras activation was 4.5% in MCF-7 cells (Fig. 6A), comparable to Ras activation in normal breast tissue and the Group A cancers; Ras activation decreased to 2% in serum-starved MCF-7 cells with EGF or serum increasing Ras activation by ~4-fold. In MDA-MB-453 cells, Ras activation was high in serum-containing medium (16%, Fig. 6B), comparable to the Group B cancers, decreased to 8% under serum starvation and increased by ~3.5- and ~1.5-fold on adding EGF or serum, respectively. In addition to overexpressing the ErbB-2 receptor, MDA-MB-453 cells also express the ErbB-3 and ErbB-4 receptors (31; 32) and, as discussed later, receptor heterodimerization between the ErbB-2 receptor and the ErbB-3 or ErbB-4 receptor may explain the cells' responsiveness to growth factors. In MDA-MB-231 cells, Ras activation was high, between 18 and 30%, changing little under all conditions (Fig. 6C); these cells, therefore, exhibited similar Ras activation as in five of the 11 Group B cancers. Thus, normal breast tissue and the Group A cancers were similar to cells without any genetic mutations and the Group B cancers were similar to ErbB-2-overexpressing cells treated with growth factors and to cells with an activating K-*ras* mutation. An anti-ErbB-2 antibody decreased Ras activation by approximately 50% in serum-stimulated MCF-7 and MDA-MB-453 cells but was without effect when the cells were serum-starved.

When we measured MAP kinase activity in the cell lines, we found that in serum-starved MCF-7 cells enzyme activity was 0.08 pmol/min/mg protein and increased ~3.5-fold with EGF; the basal activity was similar to normal breast tissue and the EGF-stimulated activity was similar to activity in the fibroadenomas and Group A cancers. MAP kinase activity in serum-starved MDA-MB-453 cells was 0.25 pmol/mg/min and increased ~1.8-fold with EGF; the EGF-stimulated values are similar to activity in the Group B cancers. MAP kinase activity in the MDA-MB-231 cells was high under basal conditions and was not influenced by growth factors. Thus, as for Ras activation, cells overexpressing the ErbB-2 receptor required growth factors for maximal MAP kinase stimulation whereas MAP kinase activity was independent of culture conditions in cells containing an activating *ras* mutation. Moreover, normal breast tissue and the Group A cancers exhibited similar MAP kinase activity as MCF-7 cells and serum-starved MDA-MB-453 cells, and the Group B cancers had similar MAP kinase activity as growth factor-treated MDA-MB-453 cells and the MDA-MB-231 cells.

DISCUSSION

Using an enzyme-based method, we found Ras was highly activated in 11 of 20 breast cancers compared to normal breast tissue; MAP kinase activity was significantly elevated in these 11 cancers compared to normal tissue. During the course of these studies, Sivaraman, et al. reported an approximate 4.5-fold increase in MAP kinase activity in 11 breast cancers compared to six benign breast lesions (16). When the Group A and Group B cancers in our cohort are combined, we also find about a 4-fold increase in MAP kinase activity relative to the normal samples. The Sivaraman group did not assess Ras activation or EGF or ErbB-2 receptor expression nor did they measure MAP kinase activation in cultured cell lines.

The mean Ras activation of 17% in the Group B cancers is likely to be of physiological significance for the following reasons. First, as already mentioned, increased Ras activation in these cancers was associated with increased MAP kinase activity. Second, we and others have found in cells transfected with a mutated, constitutively-active Ras that Ras activation is in the range of 15-30% (19; 20); this seemingly low degree of Ras activation is sufficient to transform cells by several criteria including tumorigenicity in nude mice (33; 34). And third, we have found Ras activation in the range of 20-30% in pancreatic and colonic adenocarcinomas which have activating K-*ras* mutations (unpublished observations); *ras* mutations in pancreatic and colonic carcinomas clearly play an etiologic role in the development of these cancers (35; 36). The importance of Ras activation to breast cancer cell growth was recently underscored by Stevenson *et.al.* who showed that a dominant negative Shc inhibited colony formation in several breast cancer cell lines and that an activated Ras restored the cells' growth potential (37).

Nine of the 20 breast cancers expressed the EGF receptor which is similar to that reported in the literature (7-9) but we did not find an inverse correlation between EGF receptor expression and estrogen receptor status as reported in other studies (7; 8). Ten of the 20 breast cancers expressed the ErbB-2 receptor which is higher than generally reported but can be explained by the relatively small sample size (4; 5; 12). We did not find expression of the c-FMS receptor or of the truncated constitutively-active EGF receptor, ΔEGFR, in our patient cohort; the c-FMS receptor is positive in only ~15% of breast cancers (14; 15) and the prevalence of the ΔEGFR in breast cancer is not yet well defined (13).

The data from the breast cancer cell lines related well to the data in the primary breast samples. Specifically, Ras activation in MCF-7 cells, which have no known genetic mutations in *ras* or in growth factor receptors, was in the range of that found in normal breast samples and the Group A cancers, both in logarithmically growing cells and after EGF or serum stimulation. In MDA-MB-453 cells, which overexpress the ErbB-2 receptor, Ras activation was similar to the Group B cancers, both during logarithmic growth and EGF or serum treatment. The MDA-MB-231 cells, which have an activating K-*ras* mutation, showed constitutively-elevated Ras activation unaffected by growth conditions with activation levels in the range of the Group B cancers.

Seven of the 11 cancers with increased Ras activation co-expressed the EGF and ErbB-2 receptors and the other four cancers with high Ras activation expressed one of these two receptors. The ErbB-2 receptor has no known specific ligand and it must heterodimerize with the EGF, ErbB-3 or ErbB-4 receptor to be activated (38-40). In the seven cancers co-expressing the EGF and ErbB-2 receptors, it is possible that the malignant tissue contained sufficient ligand to induce heterodimerization of these two receptors. Heterodimers of EGF and ErbB-2 can bind any of several

growth factors including EGF, amphiregulin, betacellulin, transforming growth factor-alpha (TGF-α) or heparin-binding EGF-related growth factor, all of which may play a pathogenetic role in breast cancer (41-44). In the two cancers with high Ras activation expressing only the ErbB-2 receptor, it may have dimerized with the ErbB-3 or ErbB-4 receptor; the ErbB-3 receptor shows increased expression in some breast cancers and the ErbB-4 receptor was found recently in nine of 12 breast cancers (45-47). Since only two cancers with high Ras activation expressed the ErbB-2 receptor in the absence of the EGF receptor, it did not seem indicated to assess all 20 of the malignant samples for expression of both the ErbB-3 and ErbB-4 receptors. In the two cancers with high Ras activation expressing only the EGF receptor, it is likely there was sufficiently high concentrations in the extracellular milieu of EGF, or some other EGF-like ligand, to induce receptor dimerization and, thereby, activate Ras. As is well known, overexpression of either the ErbB-2 or EGF receptor is associated with a poor clinical prognosis (4; 5; 7-9; 12) and our data suggest that the reason for a more malignant phenotype of these tumors may be because the cells are exposed to sufficient growth factors to activate Ras and MAP kinase.

Herceptin, an anti-ErbB-2 monoclonal antibody, is the newest agent in the treatment of breast cancer and its use is based partially on the premise that downstream targets of ErbB-2, e.g., Ras and MAP kinase, are activated in breast cancer (48). Our data with an anti-ErbB-2 antibody provide mechanistic support for Ras inhibition by herceptin; the finding that the antibody inhibited Ras activation only in the presence of growth factors may explain some of the variability in patient responses to herceptin. Several Ras inhibitors are in clinical trials and in animal models they are most effective in tumors exhibiting activated Ras, although their mechanism of action likely includes inhibition of other Ras-related proteins (49-53). In addition, an oncolytic reovirus has been described

which requires activated Ras for its tumorcidal properties (54). By assessing Ras activation in tumors, it should be possible to determine *a priori*, prior to initiating treatment, whether a particular cancer is likely to respond to one of these new therapeutic approaches. Since we and others have found ErbB-2 receptor expression in non-malignant breast tissue (30), and in a cancer with low Ras activation, assessing receptor expression may not be sufficient. Similarly, assessing MAP kinase activity provides information only about a single mitogenic pathway, whereas it is clear that Ras transmits mitogenic signals through several different pathways (17; 18). Moreover, the method for assessing Ras activation lends itself well to automation allowing rapid processing of multiple tumor samples. Thus, assessing Ras activation in breast cancer may have some advantages over assessing receptor expression or MAP kinase activity and may provide a basis for new treatment strategies of this disease.

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FOOTNOTES

[1]The abbreviations used are: EGF, epidermal growth factor; ΔEGFR, a truncated constitutively-active EGF receptor lacking 267 amino acids in the receptor's extracellular domain; MAP kinase, mitogen-activated protein kinase; PCR, polymerase chain reaction; FBS, fetal bovine serum.

Table I. Clinical and Pathological Data: Normal Breast Tissue and Fibroadenoma

Pt	Age	Histology	Ras Activation (%)	Other
1	50	nl breast	1.0	DCIS in other breast
2	42	nl breast	2.1	ca in other breast, NOS
3	60	nl breast	4.9	invasive ductal ca in both breasts
4	68	nl breast	5.5	invasive lobular ca in same breast
5	70	nl breast	5.6	invasive lobular ca in same breast
6	52	nl breast	5.7	DCIS in same breast
7	33	nl breast	6.8	breast reduction
8	19	fibroadenoma	3.6	
9	70	fibroadenoma	6.4	

Tables I-III. All patients were operated on at UCSD Medical Center as part of their routine clinical care. Estrogen and progesterone receptor status, the percentage of cells in S phase and chromosomal ploidy were determined as part of the clinical management of the patients according to standard procedures. For Ras activation measurements, tissue was obtained according to a procedure approved by the UCSD Institutional Review Board and was frozen within minutes of resection and analyzed as described in Materials and Methods. Abbreviations are: nl breast, benign breast tissue with or without fibrocystic changes; ca, cancer; DCIS, ductal carcinoma *in situ*; LCIS, lobular carcinoma *in situ*; NOS, not otherwise specified; LN, lymph node; ER, estrogen receptor; PR, progesterone receptor; ND, not determined; NA, not applicable, this refers to the specific site from which the sample was taken for the Ras activation measurement, not for the cancer listed under "other".

Table II. Clinical and Pathological Data: Breast Cancers, Group A

Pt	Age	Histology	Tumor size and lymph nodes	Stage	ER/PR	S-phase (%) ploidy	Ras Activation (%)	Other
10	49	LCIS	NA	NA	NA	NA	1.5	concurrent invasive lobular ca
11	53	invasive ductal	2.2 cm 1/10 LN+	IIB (T2N1M0)	+/+	8.5 tetraploid	1.9	
12	44	invasive ductal	3 cm 3/11 LN+	IIB (T2N1M0)	-/-	14.5 aneuploid	2.6	
13	65	invasive lobular, inflammatory	4 cm	IIIB (T4NxMx)	+/+	9.2 aneuploid	3.1	history of ipsilateral medullary ca
14	56	invasive ductal	1.5 cm 2/11 LN+	IIA (T1N1M0)	+/+	3.5 diploid	3.3	after neoadjuvant chemotherapy
15	62	metastatic ductal in LN	NA	NA	NA	NA	4.3	primary ca stage IIA, T1N1M0
16	66	invasive lobular	8.0 cm	IIB (T3N0M0)	+/+	ND aneuploid	6.1	
17	56	metastatic ductal in LN	NA	NA	NA	NA	6.5	primary ca stage IV, T1N2M1
18	58	invasive ductal	1.5 cm	I (T1N0M0)	-/-	14.8 aneuploid	8.5	

Table III. Clinical and Pathological Data: Breast Cancers, Group B

Pt	Age	Histology	Tumor size and lymph nodes	Stage	ER/PR	S-phase (%) ploidy	Ras Activation (%)	Other
19	52	invasive ductal	3.9 cm	IIA (T2N0M0)	+/+	9.3 aneuploid	9.8	
20	46	invasive ductal	6.3 cm 1/11 LN+	IIIA (T3N1M0)	+/+	ND	9.9	after neoadjuvant chemotherapy
21	62	invasive ductal	4.5 cm	IIB (T2N0M0)	-/-	8.8 aneuploid	12	
22	44	high grade DCIS	0.9 cm	stage 0 (Tis)	NA	NA	12	
23	48	LCIS	NA	NA	NA	NA	13	ipsilateral invasive lobular ca, 3/13 LN+
24	34	invasive ductal	3.3 cm	IIA (T2N0M0)	-/-	7.7 diploid	16	
25	41	invasive lobular	7.0 cm 2/13 LN+	III A (T3N1M0)	+/+	6.1 diploid	18	
3	60	invasive ductal	1.0 cm	I (T1NXM0)	+/-	3.1	21	see patient 3, Table I; third primary ca
26	37	invasive ductal	5.5 cm 8/15 LN+	IV (T3N1M1)	+/+	ND	23	history of metastatic ca in contralateral breast
27	49	invasive ductal	2.2 cm 2/2 LN+	IIB (T2N1M0)	+/+	9.3 aneuploid	24	
28	72	high grade DCIS	2.0 cm	stage 0 (Tis)	NA	NA	29	Paget's disease of ipsilateral nipple

FIGURE LEGENDS

Fig. 1. Characterization of Cells Analyzed For Ras Activation.

Benign breast tissue from patient number 1 (panel A) or malignant tissue from patient 11 (panel B) and patient 21 (panel C) was sectioned in the operating room and cells adherent to the scalpel blade were applied to glass slides on dry ice which were either stained with Wright's stain (panels A and B) or with an immunohistochemical stain using an anti-keratin antibody. The scale bar in all three panels is 50 μm. Note that the majority of cells are epithelial in origin, both in the benign and malignant samples.

Fig. 2. Ras Activation and EGF and ErbB-2 Expression in Patient Cohort.

Cells from resected tissue were frozen rapidly on glass slides as described in Fig. 1; Ras was immunoprecipitated from the cells and Ras activation was measured as described in Methods. Each circle represents Ras activation of one patient in the study cohort and is the mean of at least two independent measurements performed in duplicate. The wide horizontal bars show the mean value for each data set and, for the normal breast samples, the two narrow bars show two standard deviations beyond the mean. Of the 20 cancers, nine had Ras activation levels within two standard deviations of the mean value of the normal breast samples (Group A cancers) and 11 cancers had Ras activation levels greater than two standard deviations above the mean of the normal samples (Group B cancers); the mean Ras activation of the Group B cancers was significantly different from the normal samples ($p<0.05$). EGF and ErbB-2 receptor expression were assessed by immunohistochemical staining of paraffin-embedded tissue as described in Methods: open circles, negative staining for both receptors; filled circles, positive staining for both receptors; circles with

left-half filled, positive staining for EGF receptor; circles with right-half filled, positive staining for ErbB-2 receptor.

Fig. 3. Assessment for Activating Mutations in K-*ras*, Codon 12.

DNA was extracted from frozen or paraffin-embedded tissue and assessed for activating K-*ras*, codon 12 mutations as described in Methods. In each set of two lanes, the PCR product was incubated in the absence (a) or presence (b) of the restriction enzyme BstNI. Wild type K-*ras* yields a 114 bp cleavage fragment while K-*ras*, codon 12 mutations yield a 143 bp cleavage fragment; smaller fragments which were generated are not visualized. Lanes 1a & b, MCF-7 breast cancer cells which contain wild type Ras; lanes 2a & b, a human pancreatic cancer which is heterozygous for a K-*ras*, codon 12 mutation; lanes 3a & b, patient 10; lanes 4a & b, patient 15; lanes 5a & b, patient 23; lanes 6a & b, patient 26; and lanes 7a & b, patient 28.

Fig. 4. Immunohistochemical Staining for EGF and ErbB-2 Receptor Expression.

Paraffin-embedded tissue sections from the tumors of patient number 28 (panels A and B) and patient number 27 (panel C) were fixed and stained with an anti-ErbB-2 receptor antibody (panel A) or an anti-EGF receptor antibody (panels B and C) as described in Methods. The scale bar in all three panels is 50 μm. Note in panels A and B staining of the plasma membrane without cytoplasmic staining.

Fig. 5. MAP Kinase Activity in Representative Samples of Patient Cohort.

MAP kinase was immunoprecipitated from frozen cells obtained as described in Fig. 1 and MAP kinase activity was measured following phosphorylation of myelin basic protein. For each sample, the data are the mean ± SD of at least two independent measurements performed in duplicate. The designation of Group A and B cancers is as in Fig. 2.

Fig. 6. Ras Activation in Human Breast Cancer Cell Lines.

Ras activation was measured as described in Fig. 2 in three human breast cancer cell lines: A, MCF-7 cells with no known genetic mutations; B, MDA-MB-453 cells which overexpress the ErbB-2 receptor; and C, MDA-MB-453 cells with a mutation in K-*ras*, codon 12. Cells were grown in serum-containing medium (log phase), in serum-free medium for 72 h (serum starved), and in serum-free medium stimulated for 5 min with either 20% fetal bovine serum (FBS) or 100 ng/ml EGF (EGF). The data are the mean ± S.D. of at least three independent experiments performed in duplicate. Note the difference in the ordinate scale between the MCF-7 cells and the MDA-MB-453 and MDA-MB-231 cells.

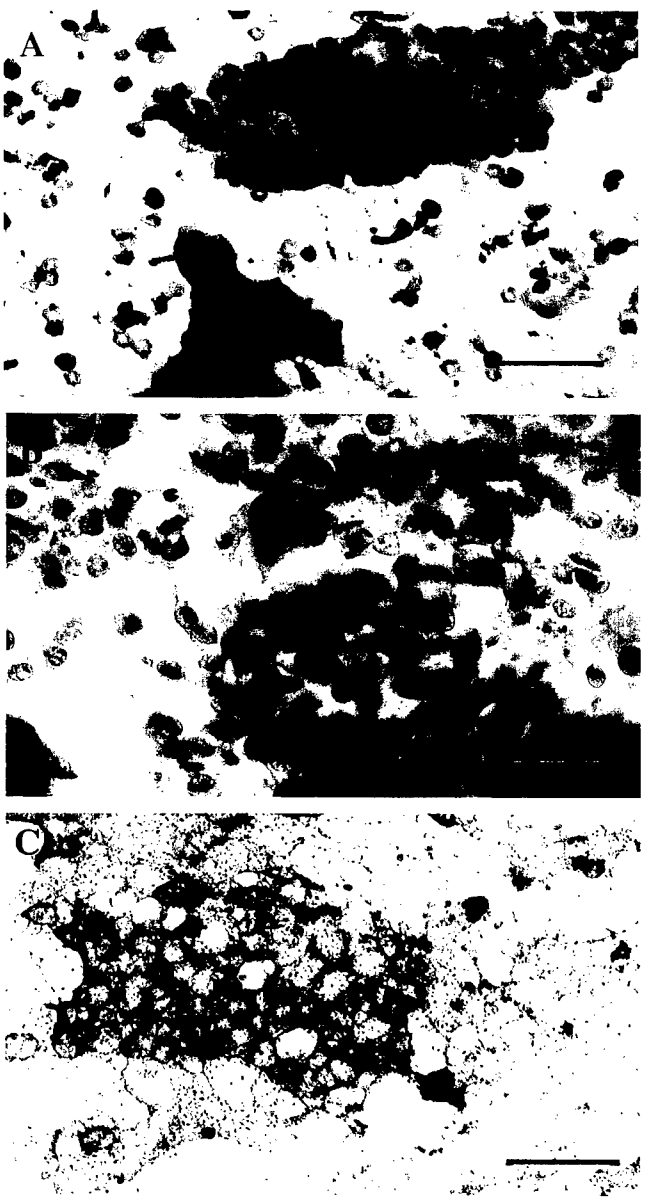


Figure 1

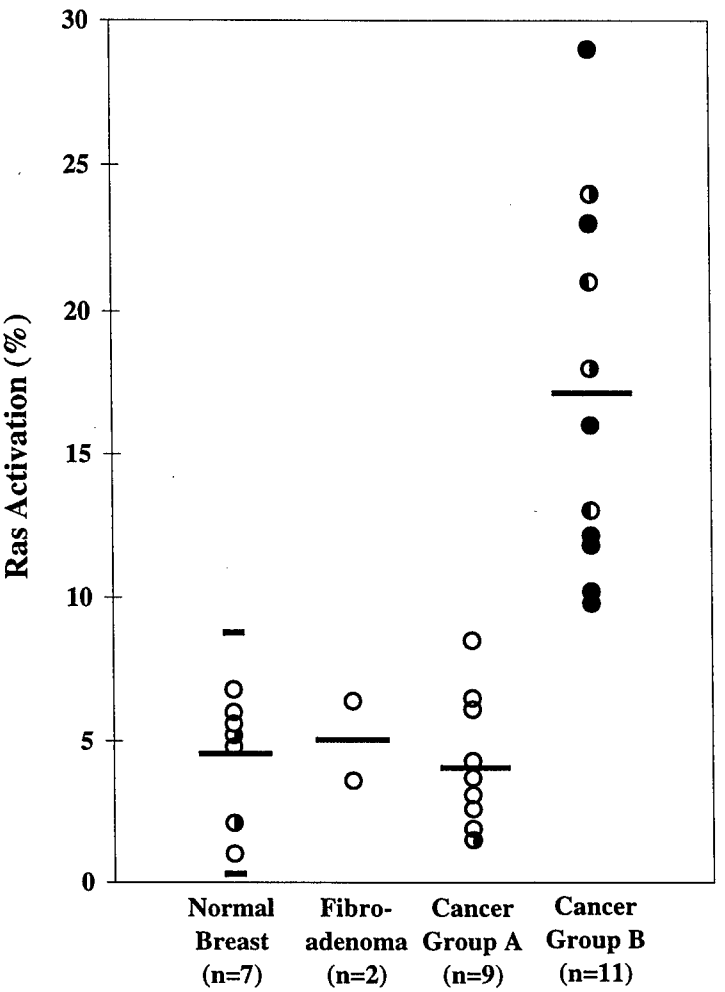

Ras Activation (%)
30
25
20
15
10
5
0
Normal Breast (n=7)
Fibro-adenoma (n=2)
Cancer Group A (n=9)
Cancer Group B (n=11)

Figure 2

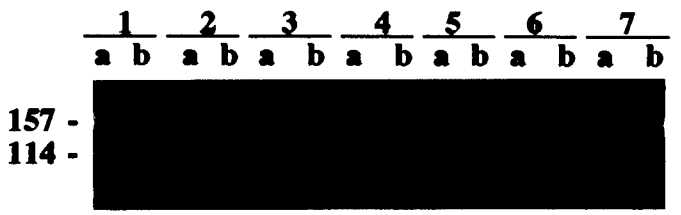


Figure 3

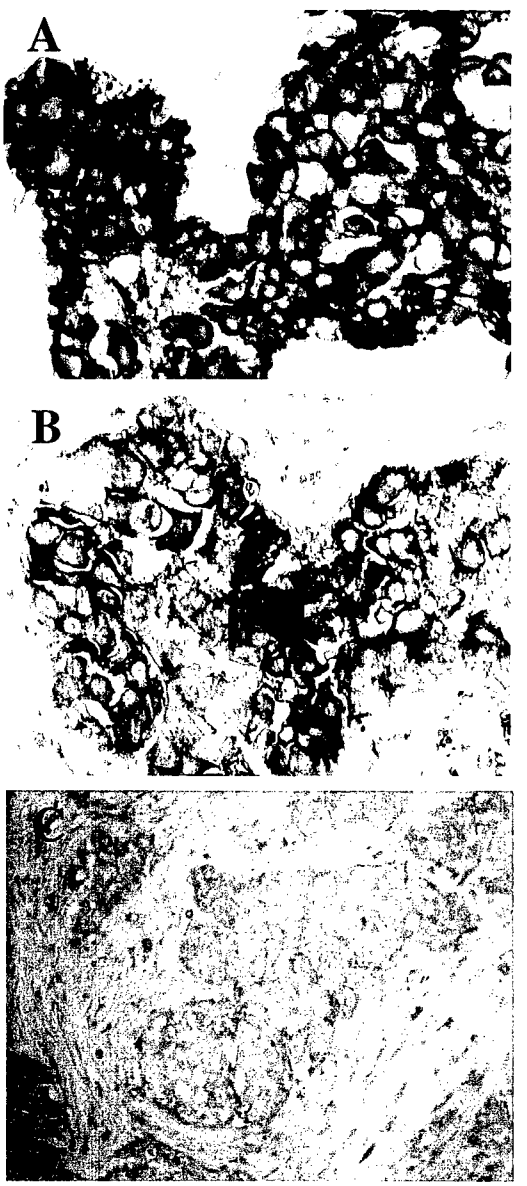


Figure 4

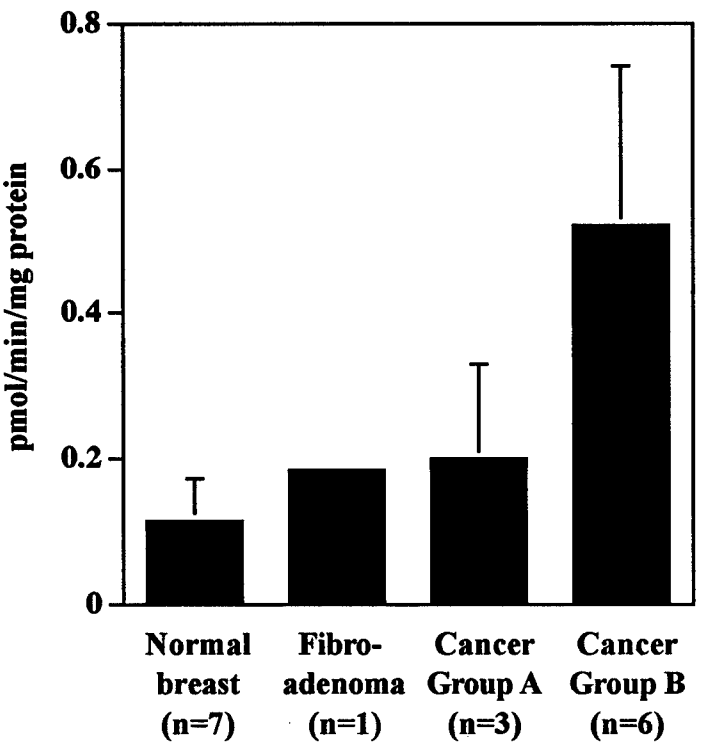
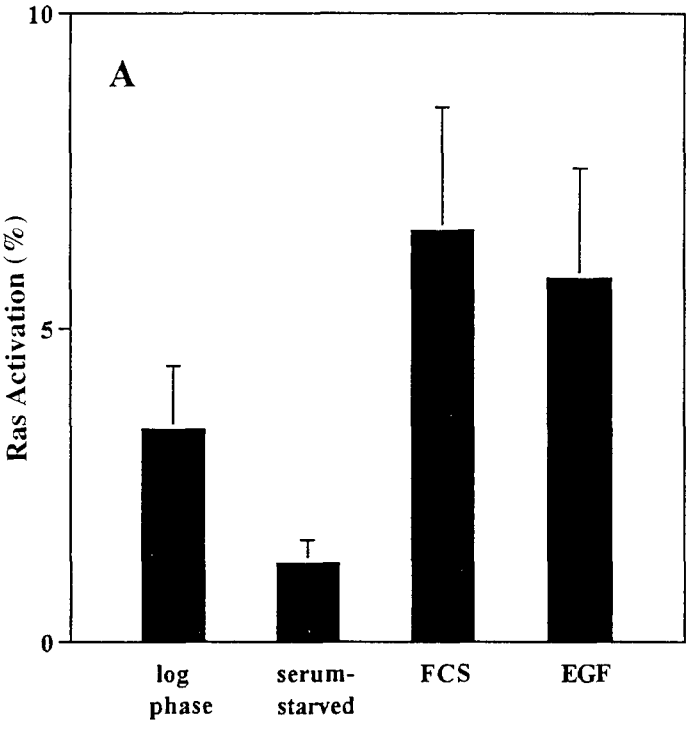
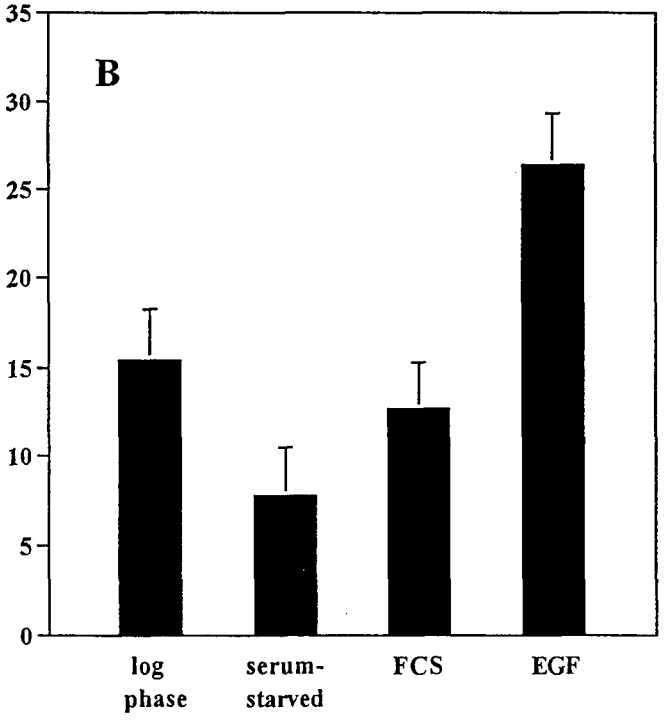

0.8
0.6
0.4
0.2
0
pmol/min/mg protein
Normal breast (n=7)
Fibro-adenoma (n=1)
Cancer Group A (n=3)
Cancer Group B (n=6)

Figure 5


A
Ras Activation (%)
10
5
0
log phase
serum-starved
FCS
EGF


B
35
30
25
20
15
10
5
0
log phase
serum-starved
FCS
EGF

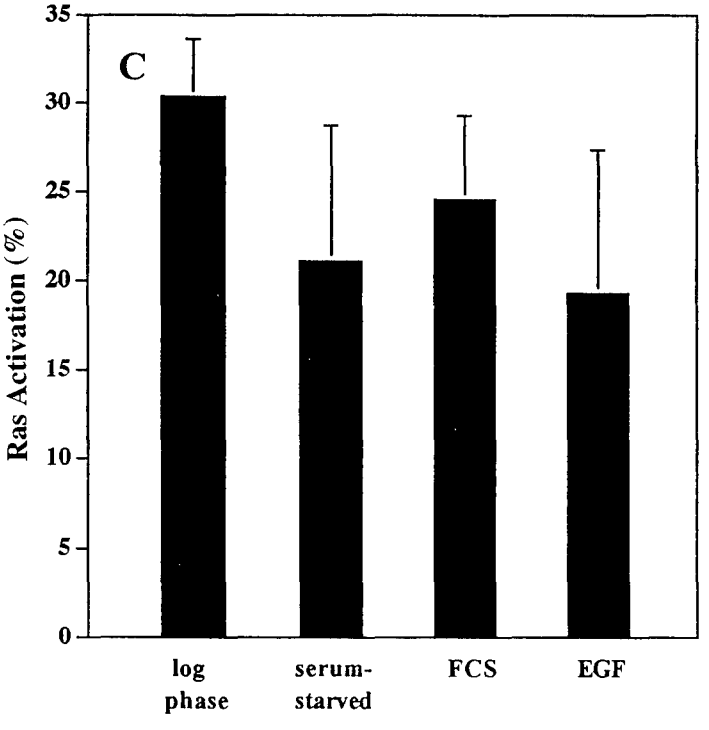

C
Ras Activation (%)
35
30
25
20
15
10
5
0
log phase
serum-starved
FCS
EGF

Figure 6